

Animal Model

Development of Hyperplasias, Preneoplasias, and Mammary Tumors in MMTV-c-erbB-2 and MMTV-TGF α Transgenic Rats

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Human cDNAs corresponding to two epidermal growth factor-related products that are overexpressed in human breast cancers, that for c-erbB-2 (HER-2) and for transforming growth factor α (TGF α), have been cloned downstream of the mouse mammary tumor virus (MMTV) long terminal repeat promoter and injected into the pronucleus of fertilized oocytes of Sprague-Dawley rats to produce transgenic offspring. Expression of the transgenic mRNAs is not detectable in mammary tissue from virgin transgenic rats but is detected in mammary tissue from certain lines of mid-pregnant transgenic rats. When two such lines of either type of transgenic rat are subjected to repeated cycles of pregnancy and lactation, they produce, primarily in the mammary glands, extensive pathologies, whereas virgin transgenic rats produce no such abnormalities. Multiparous transgenic female offspring from c-erbB-2-expressing lines develop a variety of focal hyperplastic and benign lesions that resemble lesions commonly found in human breasts. These lesions include lobular and ductal hyperplasia, fibroadenoma, cystic expansions, and papillary adenomas. More malignant lesions, including ductal carcinoma *in situ* and carcinoma, also develop stochastically at low frequency. The mammary glands of transgenic females invariably fail to involute fully after lactation. Similar phenotypes are observed in female MMTV-TGF α transgenic rats. In addition, multiparous TGF α -expressing female transgenics frequently develop severe pregnancy-dependent lactating hyperplasias as well as residual lobules of hyperplastic secretory epithelium

and genuine lactating adenomas after weaning. These transgenic rat models confirm the conclusions reached in transgenic mice that overexpression of the c-erbB-2 and TGF α genes predisposes the mammary gland to stochastic tumor development. (Am J Pathol 1999, 155:303–314)

Transgenic techniques have been used to create many models of mammary neoplasia in the mouse^{1,2} and, together with models of tissue reconstitution,³ have provided an insight into the function of various oncogenes in the development of breast cancer. With one exception,⁴ transgenic rat models for breast cancer have not yet been described. It is of great interest to extend the generation of transgenic models of breast cancer to the rat to complement and extend the results observed in the mouse. Moreover, the origin, pathology, and sensitivity to hormones of spontaneously arising rat mammary tumors closely resemble those in the human.^{5,6} Given the structural and functional resemblance of rat mammary tumors to their human counterparts, it is not surprising that experimental induction of mammary tumors in the rat by methods such as administration of carcinogens or irradiation has been carried out for many years.⁵ In contrast to these relatively imprecise methods of induction, in which the targeted gene is uncertain, creation of transgenic rats that are predisposed to breast cancer by expressing defined oncogenes in the mammary gland would enable a more controlled analysis of the pathogenesis of the disease to be carried out in this species.

Two of the most consistently expressed dominantly acting oncogenes in human breast cancer are those for transforming growth factor α (TGF α) and for c-erbB-2 (HER-2). TGF α is a member of the epidermal growth

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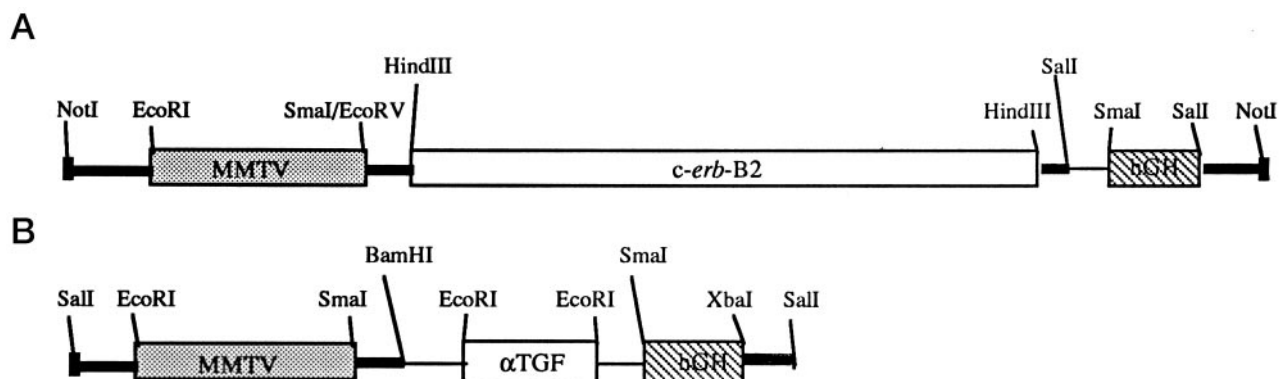


Figure 1. Diagram of the MMTV-*c-erbB-2* and MMTV-TGF α transgenes. The structure and important restriction enzyme sites used in the construction of the transgenes are shown. The MMTV-LTR promoter is used to drive expression of the downstream oncogenes *c-erbB-2* (A) and TGF α (B). A 3' fragment of the human growth hormone (hGH) gene ensures correct processing of the transcript. Thick and thin lines represent sequences derived from the polylinkers of the plasmids pPolyIII-I and pBluescript, respectively.

factor (EGF) family,⁷ and it induces a mitogenic response by activating the EGF receptor tyrosine kinase.⁸ TGF α can stimulate the growth of fibroblastic, myoepithelial-like, and epithelial cells derived from normal mammary glands and benign tumors.⁹ Moreover, under certain conditions, TGF α can mediate the growth-stimulating effects of estrogen in human breast cancer cells in culture.¹⁰ Overexpression of TGF α and its cognate receptor EGF-R have been implicated in the pathogenesis of breast cancers. Thus, minimal immunoreactive TGF α is detectable in normal human breast tissue, but increased expression occurs in ductal hyperplasia, atypical hyperplasia, and ductal carcinoma *in situ* (DCIS).¹¹ Immunoreactive TGF α has also been detected in 30% to 70% of human breast carcinomas, and its presence correlates with tumor burden.^{12–14} *c-erbB-2* also encodes a tyrosine kinase receptor^{15,16} that belongs to the EGF receptor family and is the wild-type human homologue of the transforming mutant rat oncogene *neu*.^{17,18} The level of *c-erbB-2* in normal human breast tissue is very low,¹⁹ but in invasive breast carcinomas, expression of *c-erbB-2* is observed in 20% to 30% of patients, and this enhanced expression is, in some cases, accompanied by amplification of the *c-erbB-2* gene.^{20–22} There is also an inverse correlation between *c-erbB-2* expression and patient survival, particularly in patients with no involved lymph nodes.^{23,24} Moreover, almost 50% of early x-ray-screened breast lesions of the carcinoma *in situ* type express *c-erbB-2*.²⁵ Its expression occurs in large-cell, especially comedo-type *in situ* lesions, precursors of invasive carcinoma.²⁶

In transgenic mice, the results for the transforming *c-erbB-2 neu* have been equivocal when using the mouse mammary tumor virus (MMTV) promoter to target expression of the transgene to the pregnant/lactating mammary gland. Thus, expression of *neu* in the mammary glands of transgenic mice has been reported to result in the rapid development of multifocal mammary tumors that metastasize with high frequency,^{27–29} whereas other laboratories have reported only the stochastic development of mammary tumors with little evidence of metastasis.^{30,31} Expression of the non-mutated form of the rat *c-erbB-2* gene in transgenic mice also resulted in the stochastic

development of mammary tumors, many of which metastasized.³² The reasons for these discrepancies are unclear but may depend on novel additional somatic activating mutations within *neu/c-erbB-2*.³³

In view of the potential advantages of the rat as a model for human breast cancer and in view of the discrepancies between the mouse transgenic models, we have developed a method to produce transgenic rats using the same MMTV promoter linked to the Rous sarcoma virus (RSV) long terminal repeat (LTR) enhancer to drive expression in the mammary glands of either the human TGF α or the wild-type human *c-erbB-2* genes. This approach has produced a variety of mammary hyperplasias, preneoplastic lesions, and tumors in the rat. In this communication, we report an analysis of the generation and phenotypes of these animals to the second (F₂) generation.

Materials and Methods

Construction of the MMTV Promoter-Linked Transgenes

For the *c-erbB-2* transgene, a 4.4-kb *HindIII* fragment containing the normal, unmutated human *c-erbB-2* cDNA¹⁶ was cut out of the plasmid pSV2-*erbB-2* and subcloned into the cloning vector pPolyIII-I,³⁴ to generate the plasmid pPolyIII-*erbB*. A 1548-bp *EcoRI*-*SmaI* fragment containing the RSV-LTR enhancer linked to the MMTV-LTR promoter was excised from the plasmid pMam-*neo* (Clontech, Palo Alto, CA) and cloned into the *EcoRI* and *EcoRV* sites of pPolyIII-I to generate pIII-MMTV. The *c-erbB-2* cDNA was then transferred to pIII-MMTV using the *XbaI*-*SalI* sites, to generate the plasmid pIII-MMTV-*erbB*. A 667-bp *SmaI*-*XbaI* fragment containing a splice and polyadenylation signal from the 3' end of the human growth hormone (hGH) gene was excised from the plasmid pBShGH (a gift from Dr. J. Gordon, Washington University, St. Louis, MO) and subcloned into the *SmaI*-*XbaI* sites of pBluescriptKS[–] to generate the plasmid pBlue-hGH3'. To complete the transgenic construct, the

hGH3' sequence was transferred from pBlue-hGH3' to pIII-MMTV-*erb* by digesting with *SaI* (Figure 1a).

For the MMTV-TGF α transgene, a 925-bp *EcoRI* fragment containing the unprocessed 917-bp TGF α cDNA sequence was cut out of the plasmid pHGF1-10-925 (a gift from Dr G. Bell, University of Chicago, Chicago, IL) and cloned into pPolyIII-I to generate the plasmid pPolyIII-TGF α . The same 667-bp *SmaI*-*XbaI* fragment containing the splice and polyadenylation signals as above was cloned into the *SmaI*-*XbaI* sites of pPolyIII-TGF α to generate the plasmid pPolyIII-TGF α -hGH3'. The same 1548-bp *EcoRI*-*SmaI* fragment containing the enhancer and MMTV-promoter as above was then cloned into the *EcoRI* and *SmaI* sites of pBluescriptKS⁻ to generate pBlue-MMTV. To complete the transgenic construct, a *Bam*HI-*XbaI* fragment from pPolyIII-TGF α -hGH3' was cloned into the *Bam*HI and *XbaI* sites of pBlue-MMTV to generate pBlueMMTV-TGF α -hGH3' (Figure 1b). The identities of both plasmids were confirmed by restriction endonuclease mapping, and the correct nature of the ligation points was confirmed by dideoxy chain termination DNA sequencing. The completed transgenes MMTV-*erb*-hGH3' and MMTV-TGF α -hGH3' were released from their parental plasmids before being microinjected by digestion with *NotI* and *SaI*, respectively (Figure 1).

Generation and Screening of Transgenic Rats

All animals were maintained and procedures were performed in accordance with the British Home Office Animals (Scientific Procedures) Act 1986, under Project License 80/00733. Transgenic rats were generated by pronuclear injection of linearized DNA into fertilized eggs of Sprague-Dawley rats (Charles River Laboratories, Kent, UK), as previously described for transgenic mice³⁵ with the following modification. The female rats were superovulated at 30 days of age by continuous infusion rather than an injection of purified porcine pituitary follicle-stimulating hormone (FSH; VetrepHarm, London, Ontario, Canada) via Alzet miniosmotic pumps (Alzet model 2001, Alza Scientific Products, Palo Alto, CA)³⁶. Each pump was filled with 200 μ L of FSH diluted in sterile saline and was inserted intraperitoneally into pentobarbital-anesthetized animals 2 days before mating. Synchronization of ovulation was induced 48 to 52 hours later by an intraperitoneal injection of 100 ng of luteinizing-hormone-releasing hormone analogue (des-gly10(D-alA)-LHRH-ethylamide, Sigma Chemical Co., Poole, UK). After being mated overnight, the females with vaginal plugs were sacrificed by cervical dislocation. The pumps were transferred to a second set of animals, and embryos were collected in Dulbecco's phosphate-buffered saline (PBS) from the oviducts of plugged females. Embryos were rinsed free of cumulus cells in 0.1% (w/v) hyaluronidase and transferred to modified M2 medium for microinjection or modified M16 medium (280 mOsm) for culture at 38.5°C in 5% (v/v) CO₂ until pronuclei became distinguishable.³⁵

Pronuclear injections were performed on a Nikon inverted microscope equipped with Narishige microma-

nipulators and Normarski optics. The excised DNA constructs were injected at approximately 2 ng/ μ L in 10 mmol/L Tris/HCl, 0.1 mmol/L EDTA, pH 7.4.³⁷ After injection of one pronucleus in each embryo (as evidenced by pronuclear expansion), all embryos were incubated in modified M16 until transfer. Epinephrine at 0.1% (w/v) was applied to the ovarian bursa of pseudopregnant recipients, and the bursa was torn to allow access to the infundibulum. The embryos were then transferred bilaterally to the oviduct using a finely drawn glass pipette³⁸ either into day 1 pseudopregnant recipients (synchronous) or, after overnight culture, at the early two-cell stage into day 1 (asynchronous) or day 2 (synchronous) recipients. Transgenic founder rats and subsequent transgenic offspring were identified by Southern blot analysis of genomic DNA from tails of F1 litters at 10 days of age.³⁵ Ten micrograms of appropriate restriction-enzyme-digested tail tip DNA was fractionated on 0.8% (w/v) agarose gels, transferred to Hybond N⁺ membranes (Amersham International, Little Chalfont, UK) by blotting in 0.4 mol/L NaOH and hybridized with the human *c-erbB-2* or human TGF α cDNA probe labeled by random incorporation of [³²P]dCTP (random primed DNA labeling kit, Boehringer, Mannheim, Germany) to a specific activity of 0.5×10^9 to 1×10^9 dpm/ μ g³⁹ and used to screen for transgene-positive rats. Transgene copy number was estimated by comparison with copy number controls.

Detection of Transgene Transcripts

Total RNA was isolated from tissues and tumors using the guanidinium isothiocyanate/cesium chloride method.³⁹ Poly(A)-containing RNA was isolated using the PolyAtract mRNA isolation system (Promega, Madison, WI). For RNA hybridizations, 10- μ g samples of poly(A)-containing RNA were subjected to denaturing-gel electrophoresis using formaldehyde³⁹ and transferred to nylon filters (Hybond N⁺). Hybridization and washing conditions were carried out according to the manufacturer's instructions (Amersham). Filters were subsequently hybridized using the above cDNA probes under the same conditions to a cloned cDNA corresponding to non-muscle actin to ensure consistency of loading between lanes. The radioactively hybridized filters were exposed to x-ray film, and those lanes containing different mRNA preparations were scored positive if a band corresponding to the correct sized mRNAs for *c-erbB-2* or TGF α was detected. A preparation of mRNA from MCF-7 cells⁴⁰ was used as a positive control.

Whole-Mount Analysis

Mammary glands were dissected from the skin, mounted on glass slides, fixed overnight in Methacarn (60% (v/v) methanol, 30% (v/v) 1,1,1-trichloroethane, 10% (v/v) glacial acetic acid) and then stained with carmine.⁴¹ Macroscopically identifiable tumors were cut out and processed for histology separately. Glands were cleared by immersing in methyl salicylate. Whole mounts were pho-

Table 1. Generation of MMTV-*c-erbB-2* and MMTV-TGF α Transgenic Rats

Founder	Sex	Copies of transgene	Inheritance	Transgene mRNA
MMTV- <i>c-erbB-2</i>				
ERB/1	F	10–20	Mendelian	+
ERB/2	F	1–5	Mendelian	+
ERB/3	F	~50	Mendelian	+
ERB/4	M	1 and >20*	Mendelian	–
ERB/5	F	<1	Unknown [†]	–
ERB/6	F	5–10	Mosaic	–
ERB/7	M	1	Sex-linked [‡]	–
MMTV-TGF α				
TGF/1	F	5–10	Mendelian	+
TGF/2	F	1	Mosaic	+
TGF/3	M	10	Mosaic	–
TGF/4	M	10	Mosaic	–
TGF/5	M	5–10	No transmission	–
TGF/6	M	1–5	Unknown [§]	–

Founder indicates transgenic rat produced from either the MMTV-*erb*-hGH3' or MMTV-TGF α -hGH3' constructs. Copies of transgene were determined by Southern hybridizations to tail-tip DNA in relation to 1, 10, and 100 copy DNA controls (Materials and Methods). Inheritance was either Mendelian or mosaic occurring in less than 50% of the F1 offspring, but thereafter inheritance was Mendelian in the F2 generation. Transgene mRNA was determined by Northern hybridizations in mammary tissue from 11-day pregnant female offspring: +, detectable; –, not detectable by autoradiography (Materials and Methods). F, female; M, male.

*Two independent integration sites.

[†]Sub-fertile, all the rats from the single litter were negative for the transgene.

[‡]Transgene on Y chromosome, no female transgenic offspring produced.

[§]Could not be successfully mated.

tographed using Ilford Technical Pan film. Areas of interest and suspect lesions were cut out of the whole mount and processed for histology.

Histology

Samples of mammary glands and any suspect lesions, including tumors, were fixed overnight in Methacarn, dehydrated in 70% (v/v) ethanol, and embedded in paraffin wax at 60°C on a Tissue Tek III embedding center (Miles, Slough, UK). Precooled blocks at –20°C were sectioned at room temperature on an Anglia AS 300 rotary microtome (Raymond A. Lamb, London, UK) using stainless steel disposable blades (Raymond A. Lamb). Sections 2 μ m thick were transferred onto glass microscope slides that had been pretreated with glycerin albumin (Raymond A. Lamb) and incubated for at least 1 hour at 60°C. Sections were stained with hematoxylin and eosin (H&E). Details were recorded from at least two sections of each mammary gland/lesion. Photographs were recorded on Ilford Pan F film. Slides were examined by two independent observers (B.R. Davies and P.S. Rudland), and pathology was diagnosed as defined by the UK Royal College of Pathologists Working Group.⁴²

Immunocytochemistry

Immunocytochemical staining of tissue sections was carried out using an antibody complex/horseradish peroxidase method (Dako, High Wycombe, UK).⁴³ Sections were rehydrated, treated with 0.5% (v/v) H₂O₂ in methanol to remove endogenous peroxidase and then with 0.5% (w/v) saponin for 30 minutes, and washed in water and then in PBS. They were then incubated with either a mouse monoclonal antibody to human TGF α (Oncogene Science, Cambridge, UK) or a rabbit polyclonal antibody

to a human *c-erbB-2* peptide (Dako), for 1 hour at room temperature. Sections were subsequently incubated with second antibody (biotinylated rabbit anti-mouse IgG or goat anti-rabbit IgG; Dako) and then with the streptavidin-biotin horseradish peroxidase complex, and finally, the color was developed with diaminobenzidine in H₂O₂. Selected sections were also stained with rabbit polyclonal antibodies to human callus keratin, chick smooth muscle actin, and mouse laminin, as previously described.⁴⁴ Sections were counterstained using Mayers hemalum. The percentage of immunocytochemically stained cells was verified from five randomly chosen fields from at least two sections of each of the mammary lesions by two independent observers. The specificity of staining for each antibody was checked by the complete abolition of immunocytochemical staining without the first antibody and by previous incubation of the first antibody with preparations of the requisite antigen.⁴⁴ Photographs were recorded in a Reichert-Polyvar microscope on Ilford Pan F film.

Results

Generation of MMTV-TGF α and MMTV-*c-erbB-2* Transgenic Rats and Selection of Transgenic Lines

Hybridizations of the human *c-erbB-2* and TGF α cDNAs to tail-tip DNAs indicated that 7/105 (7%) and 6/42 (14%) of the offspring resulting from oviduct transfers of MMTV-*c-erbB-2*- and MMTV-TGF α -microinjected embryos, respectively, were transgenic. The founder animals contained from less than 1 up to 50 copies of the integrated transgene per haploid genome (Table 1) and, where successfully detected, transmitted the transgene either in

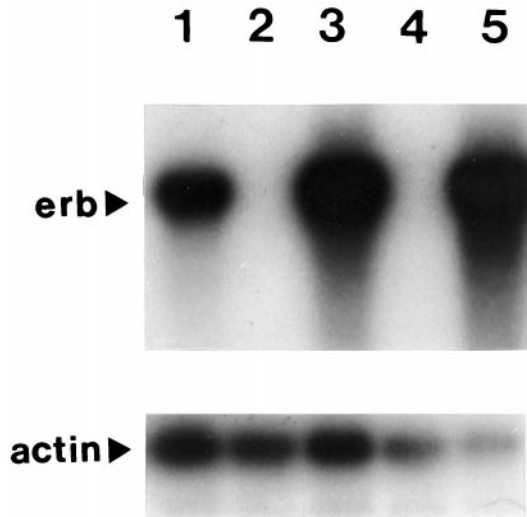


Figure 2. Northern Blot for human *c-erbB-2* mRNA in mammary tissue and lesions. **Lane 1**, 11-day mid-pregnant transgenic rat; **lane 2**, virgin transgenic rat; **lane 3**, fibroadenoma in transgenic rat; **lane 4**, 11-day mid-pregnant, nontransgenic rat; **lane 5**, carcinoma in transgenic rat.

Mendelian or mosaic fashion to their F1 generation. Thereafter, transmission, if it occurred, was exclusively Mendelian (Table 1). The results were confirmed by hybridizing a probe coding for the MMTV promoter to the same tail-tip DNA (not shown).

Transcripts of either transgenic mRNAs were not detectable in mammary tissue from virgin transgenic rats but were detected in mammary tissue from 11-day pregnant females (eg, Figure 2) in three lines of MMTV-*c-erbB-2* transgenics and in two lines of MMTV-TGF α transgenics (Table 1). Two lines of the MMTV-*c-erbB-2*-expressing transgenics (designated ERB/1 and ERB/2) and the two lines of MMTV-TGF α -expressing transgenics (designated TGF/1 and TGF/2) were selected for intensive breeding. As expression of the MMTV-*c-erbB-2* and MMTV-TGF α transgenes appeared to be induced during pregnancy, it was decided to subject the majority of the females to repeated cycles of pregnancy and lactation to maximize expression of the transgene. A total of 23 females for the *c-erbB-2*-expressing and 29 females for the TGF α -expressing transgene were observed for a period of up to 18 months, and then (or earlier if tumors developed), the mammary glands were whole mounted and examined.

Mammary Lesions in MMTV-*c-erbB-2* Transgenic Rats

No tumors appeared in the mammary glands of virgin transgenic females, and histologically their mammary glands resembled those of normal wild-type rats. The most common lesion to develop in multiparous transgenic rats was fibroadenoma in 15/23, or 65%, of the animals (Table 2). Large, macroscopically visible fibroadenomas developed in 4 multiparous animals, and multiple foci of densely staining mammary tissue were found in whole mounts in another 11 animals at autopsy, which

Table 2. Mammary Phenotypes in MMTV-*c-erbB-2* Transgenic Rats

Tissue/lesion	Frequency (%)	Immunocytochemical staining for <i>c-erbB-2</i>
Mid-pregnant glands	23/23 (100%)	+
Hyperplasia	18/23 (78%)	+ / ++
Cystic expansion	4/23 (17%)	+++
Fibroadenoma	15/23 (65%)	++
Papillary adenoma	3/23 (13%)	+
DCIS	3/23 (13%)	+
Carcinoma	2/23 (9%)	+++

All lesions arose in multiparous female rats. Frequency is expressed as the number of animals with tissue or lesion/total number of animals analyzed, with the percentages in parentheses. Immunocytochemical staining of epithelial cells was classified as negative (–), weak (+), moderate (++), or strong (+++), corresponding to the intensity of staining; myoepithelial cells and fibroblasts were unstained.

on histological examination were found to be small fibroadenomas or fibroadenomatous changes (Figure 3a). These lesions were frequently observed in close association with ductal hyperplasia of usual type or sclerosing adenosis. The epithelial cells in these fibroadenomas were immunocytochemically stained moderately on their cell membranes by antiserum to *c-erbB-2* (25% to 50% of the cells stained; Figure 3b). Areas of thickened ducts were observed in 4/23, or 17%, of animals; these were found to be due to large, focal cystic changes (Figure 3c). The epithelial cells in these cystic expansions were immunocytochemically stained strongly by antiserum to *c-erbB-2* (50% to 100% of cells stained), whereas the surrounding normal ducts either failed to stain or stained very weakly (<5% of cells stained; Figure 3d). Focal areas of hyperplasia (enlarged lobules) were observed in 18/23, or 78%, of animals at autopsy (Table 2). Large hyperplastic lobules were commonly observed that resembled normal lactating mammary gland, with secretions present in the lumen of the ducts even after 6 months or more had elapsed since lactation, whereas these secretions were no longer apparent in the equivalent mammary glands from nontransgenic rats. These hyperplastic lobules were weakly to moderately stained by antiserum to *c-erbB-2* (5% to 50% of cells stained; Table 2).

In addition to the fibroadenomas, other types of tumors developed in multiparous animals but at a significantly lower frequency (Table 2). These included tumors of mixed histological appearance but consisting mainly of papillary epithelium within cystic spaces in 3/23, or 13%, of the animals (Figure 3e). These tumors were classified as benign papillary adenomas because they were non-invasive and cytologically benign and retained myoepithelial cells and an intact basement membrane, as determined by immunocytochemical staining for smooth muscle actin and for laminin, respectively. Localized areas of more malignant looking pleomorphic epithelium were observed within or adjacent to each of these tumors (Figure 3f). These areas resembled ductal carcinoma *in situ* (DCIS) and were localized and non-invasive and were still circumscribed by myoepithelial cells and an intact basement membrane. The epithelial cells in areas of papillary adenoma and DCIS were both immunocyto-

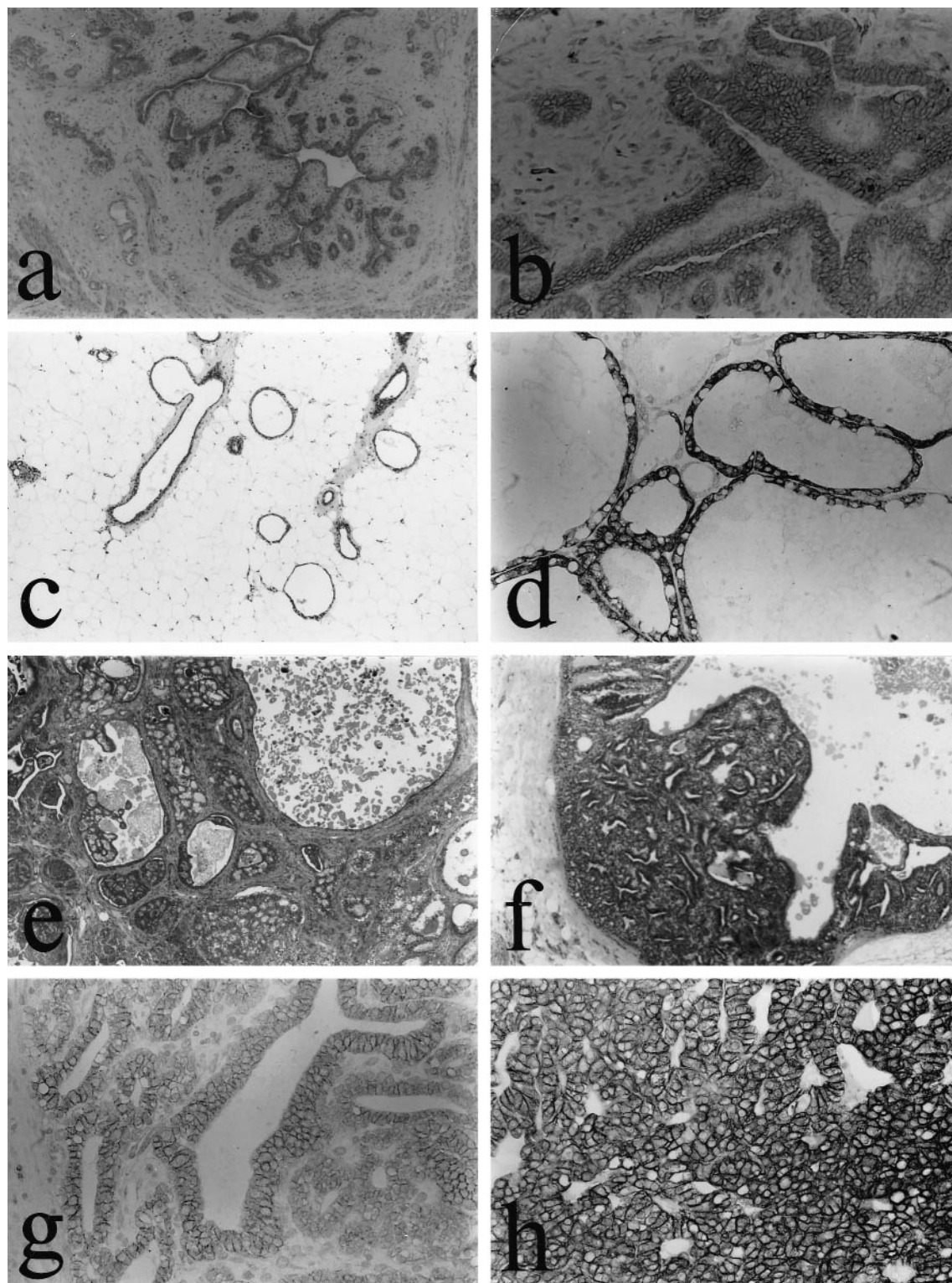


Figure 3. Histology and immunocytochemistry of mammary lesions in MMTV *c-erbB-2* transgenic rats. **a:** Fibroadenoma stained with H&E. **b:** Fibroadenoma immunocytochemically stained with antiserum to *C-erbB-2*. **c:** Area of cystic expansions stained with H&E. **d:** Cystic expansions immunocytochemically stained with antiserum to *c-erbB-2*. **e:** Papillary adenoma stained with H&E. **f:** Ductal carcinoma *in situ* (DCIS) stained with H&E. **g:** DCIS immunocytochemically stained with antiserum to *c-erbB-2*. **h:** Carcinoma immunocytochemically stained with antiserum to *c-erbB-2*. Magnification, $\times 10$ (**a**, **c**, **e**, and **f**) and $\times 40$ (**b**, **d**, **g**, and **h**).

chemically stained weakly with antiserum to *c-erbB-2* (5% to 25% of cells stained; Figure 3g). Two of twenty-three, or 9%, of animals developed rapidly growing, solid tumors after multiple pregnancies and more than 1 year of

life. These tumors were predominantly epithelial in that they could be stained by antisera to human callus keratin (not shown). They were classified as well differentiated carcinomas, as they were locally invasive, they lacked

Table 3. Mammary Phenotypes in MMTV-TGF α Transgenic Rats

Tissue/lesion	Frequency (%)	Immunocytochemical staining for TGF α
Mid-pregnant glands	29/29 (100%)	+
Severely hyperplastic lumps*	11/29 (38%)	++/+++
Hyperplasia (non-pregnant gland)	21/21 [†] (100%)	++/+++
Fibroma	1/29 (3%)	+++ [‡]
Papillary ductal adenoma	3/29 (10%)	++
Lactating adenoma	2/29 (7%)	++/+++
DCIS	4/29 (14%)	+ [§]
Carcinoma	2/29 (7%)	-(+++) [¶]

All lesions arose in multiparous female rats. Frequency is expressed as the number of animals with tissue or lesion/total number of animals analyzed, with the percentages in parentheses. Immunocytochemical staining was classified as negative (-), weak (+), moderate (++), or strong (+++), corresponding to <5%, 5% to 25%, 25% to 50%, or 50% to 100% of the epithelial cells stained; myoepithelial cells and fibroblasts were unstained.

*Occurred only in pregnant glands.

[†]Only 21 rats were examined because 8 were culled due to severe hyperplasia during pregnancy.

[‡]Fibroblasts only stained.

[§]Focal staining of epithelial cells.

[¶]Largely negative but strong staining in squamous areas.

myoepithelial cells, and the basement membrane was either very fragmented or absent, as determined above. These tumors were immunocytochemically stained very intensely with antiserum to *c-erbB-2* (50% to 100% of cells stained; Figure 3h), whereas the epithelial cells in normal ducts adjacent to the tumor failed to stain. There was no evidence of metastases in lungs, lymph nodes, or any other tissue examined. The expression of the *c-erbB-2* transgene detected by immunocytochemistry was reflected in the detection of the corresponding mRNA in fibroadenomas and carcinomas at levels above those in mid-pregnant mammary tissue from transgenic rats (Figure 2).

Mammary Lesions in MMTV-TGF α Transgenic Rats

No tumors appeared in the mammary glands of virgin TGF α transgenic females, and histologically their mammary glands were normal. The most striking phenotype observed in both lines of multiparous female MMTV-TGF α transgenics was the development of large, solid, palpable lumps in the mammary glands during pregnancy. These lumps first appeared after five or more pregnancies in 11/29, or 38%, of transgenic rats, all within the first year of life (Table 3). Sometimes, a single mammary gland was involved, but more commonly lumps developed in several glands. In the most severe cases, lumps developed bilaterally throughout the entire mammary tree and grew up to 5 cm in diameter, necessitating culling of eight animals. The lumps always appeared on the 10th or 11th day of pregnancy but invariably regressed the day before birth, allowing the animals to lactate normally and

nurse their pups. However, these lumps then reappeared, usually more severely during the next pregnancy. On histological examination these lesions consisted of solid masses of tissue resembling normal lactating mammary gland (Figure 4, a-c); their growth characteristics and histology suggested that they were not neoplasias, but severe hyperplasias. An increased number of lobules and an increased size of individual lobules were apparent. The hyperplastic mammary tissue compressed the surrounding normal fat and muscle tissue but did not invade. At least 70% of the epithelial cells in these hyperplasias stained strongly with antiserum to TGF α , predominantly in the cytoplasm (Figure 4d). Stromal cells, where present, failed to stain. Whole-mounted mammary glands from transgenic females during earlier pregnancies, where these macroscopically visible hyperplasias were not apparent, revealed that the mammary tissue was also hyperplastic and capable of being stained with antiserum to TGF α to a similar extent (Table 3). Frequently, the fat pad was completely filled with proliferating mammary epithelium, and individual lobules merged with one another. This was not the case in non-transgenic littermates where residual fatty tissue remained and individual lobules could frequently be distinguished (Figure 4b).

Whole mounts of involuted mammary glands from the remaining 21 multiparous transgenic females (more than eight pregnancy/lactation cycles) at least 6 weeks after weaning their last litter revealed extensive retention of densely staining foci in the regressed glands (100% surviving rats). Histological examination showed that these foci consisted of residual lobules of hyperplastic secretory epithelium (Figure 4j), whereas glands from control littermates consisted of only small, condensed ducts/alveolar lobules with no evidence of lactation. Areas of mammary hyperplasia in transgenic females were immunocytochemically stained moderately to strongly in their cytoplasm with antiserum to TGF α (25% to 100% of cells stained; Table 3), whereas adjacent normal involuted lobules generally failed to stain (<5% of cells stained; not shown). For example, a very densely staining area of mammary tissue 3 mm in diameter was found at the edge of a whole-mounted inguinal gland from the TGF/1 founder animal. On histological analysis, this consisted of an area of dense hyperplasia, including sclerosing adenosis, with elements of fibroadenoma also present (Figure 4j); the latter elements were unstained by antiserum to TGF α (<5% of cells stained; not shown).

Palpable tumors that grew progressively larger, independent of pregnancy, also developed after multiple pregnancy/lactation cycles in 8/29, or 28%, of the female transgenics. The individual types, however, occurred at a significantly lower frequency than the hyperplasias (Table 3). These tumors included a fibroma, papillary adenomas, lactating adenomas, DCIS, and carcinoma (Figure 4, e and f). The carcinomas were poorly differentiated with a mixture of elongated/spindle cells, DCIS, and carcinomatous elements, which frequently showed areas of squamous and sebaceous gland-like differentiation (Figure 4e). The squamous areas usually surrounded cystic spaces (Figure 4f). Weak focal cytoplasmic staining for

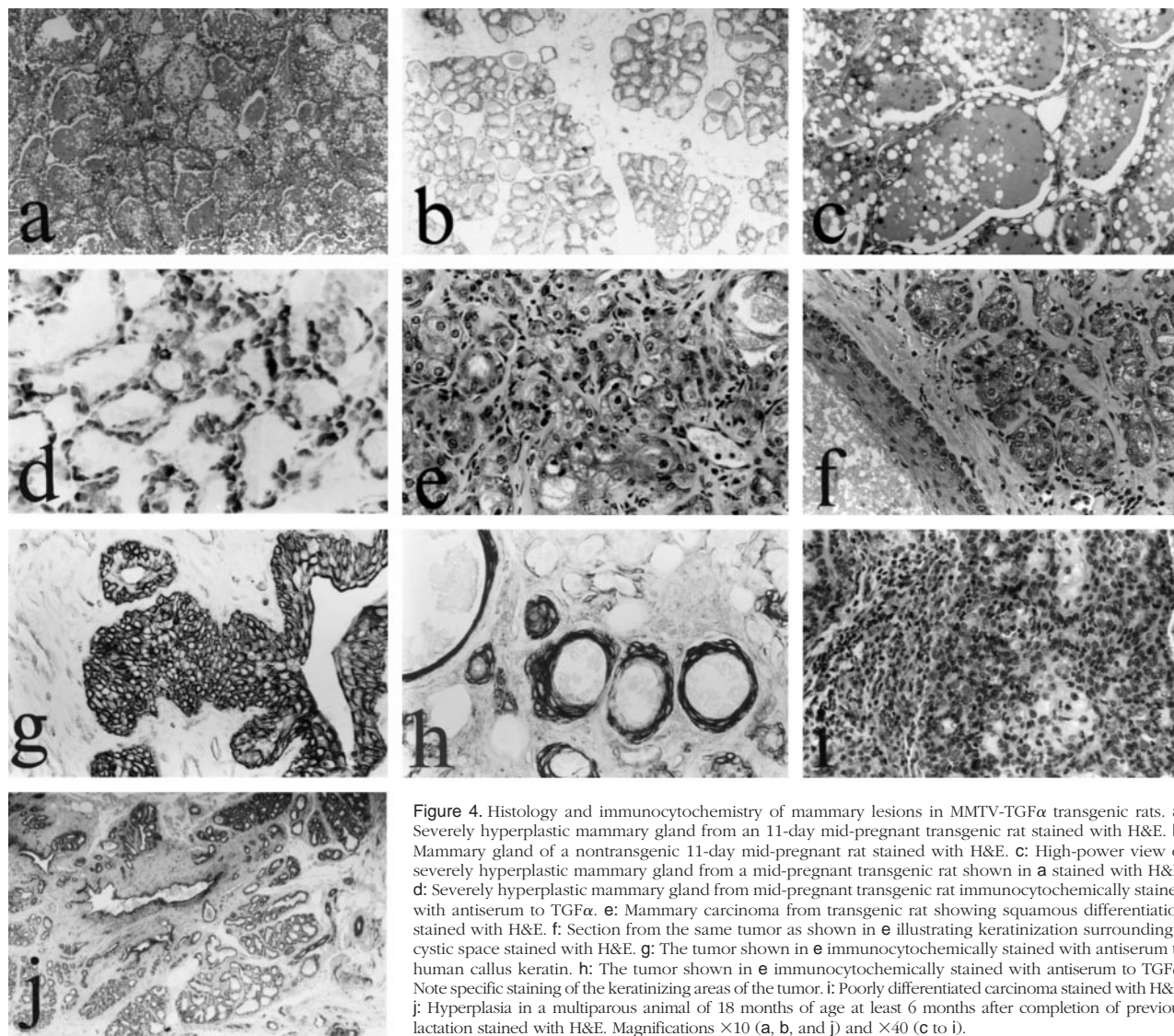


Figure 4. Histology and immunocytochemistry of mammary lesions in MMTV-TGF α transgenic rats. **a:** Severely hyperplastic mammary gland from an 11-day mid-pregnant transgenic rat stained with H&E. **b:** Mammary gland of a nontransgenic 11-day mid-pregnant rat stained with H&E. **c:** High-power view of severely hyperplastic mammary gland from a mid-pregnant transgenic rat shown in **a** stained with H&E. **d:** Severely hyperplastic mammary gland from mid-pregnant transgenic rat immunocytochemically stained with antiserum to TGF α . **e:** Mammary carcinoma from transgenic rat showing squamous differentiation stained with H&E. **f:** Section from the same tumor as shown in **e** illustrating keratinization surrounding a cystic space stained with H&E. **g:** The tumor shown in **e** immunocytochemically stained with antiserum to human callus keratin. **h:** The tumor shown in **e** immunocytochemically stained with antiserum to TGF α . Note specific staining of the keratinizing areas of the tumor. **i:** Poorly differentiated carcinoma stained with H&E. **j:** Hyperplasia in a multiparous animal of 18 months of age at least 6 months after completion of previous lactation stained with H&E. Magnifications $\times 10$ (**a**, **b**, and **j**) and $\times 40$ (**c** to **i**).

TGF α was usually observed in areas of DCIS (5% to 25% of cells stained), but in adjacent carcinomatous areas, staining for TGF α was either very weak or absent ($<5\%$ of cells stained; Table 3). However, where metaplastic differentiation to squamous elements occurred, as evidenced by staining for callus keratins (Figure 4g), TGF α immunostaining was uniformly strong (50% to 100% of cells stained; Figure 4h). Some carcinomatous areas were very anaplastic. Hyperplasia of usual type and adenomatous areas were also present in the breast tissue surrounding the carcinomas.

Other Phenotypes in Transgenic and Nontransgenic Rats

Loss of fur was observed in male and female MMTV-TGF α transgenic rats in both the TGF/1 and TGF/2 lines. This loss of fur was not uniform in terms of time of onset and location. Transverse sections through the skin in areas of hair loss revealed hyperplasia of the sebaceous

glands. The sebaceous glands were immunocytochemically stained strongly with antiserum to TGF α (50% to 100% of cells stained; Figure 5, a and b). Other components of the dermis failed to stain with antiserum to TGF α . Although the transgenes were weakly expressed in various other tissues of certain transgenic lines, including epithelial cells of the male reproductive tracts, spleen, and salivary glands, no abnormal phenotypes were observed in these tissues with the exception of the salivary glands where hyperplasia sometimes occurred in both MMTV-*c-erbB-2* and MMTV-TGF α transgenic rat lines (not shown).

Twenty nontransgenic littermates of similar age and reproductive histories (mean, 18 months and nine pregnancies) were also examined to check for the development of spontaneously arising mammary and other lesions. A single area of ductal hyperplasia of usual type and a small area of fibroadenoma were found in the mammary glands of two separate animals, and the remainder showed no gross or histologically detectable

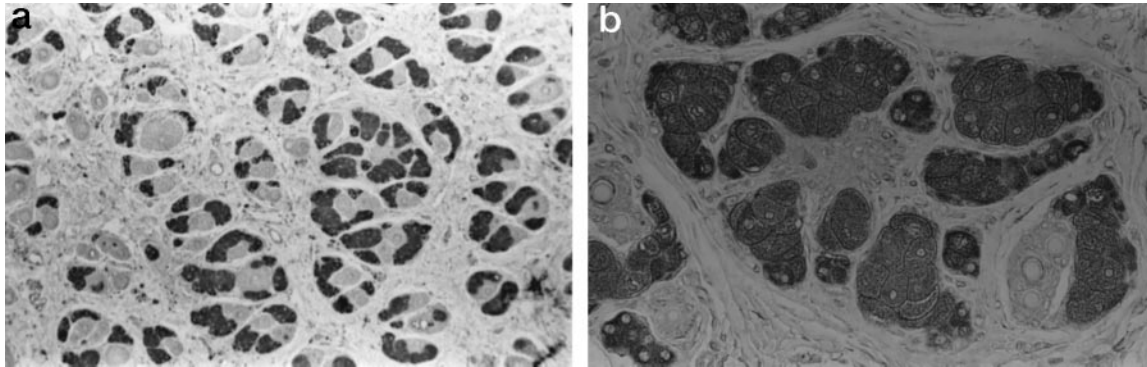


Figure 5. Sebaceous gland hyperplasia in MMTV-TGF α transgenic rats. **a:** Sebaceous gland hyperplasia in the dermis of the skin of a TGF/1 female transgenic rat immunocytochemically stained with antiserum to TGF α . The sebaceous glands stained intensely whereas other structures failed to stain. **b:** High-power detail of the section shown in **a**. Sebaceous glands of nontransgenic control rats failed to stain with the same anti-TGF α antiserum (not shown). Magnification, $\times 10$ (**a**) and $\times 40$ (**b**).

mammary or other lesions. Neither these lesions nor normal mammary tissue were stained by antiserum to *c-erbB-2* or TGF α (not shown).

Discussion

In this report we have described the generation and analysis of the mammary phenotypes of transgenic rats expressing the human *c-erbB-2* and TGF α genes under the control of the MMTV-LTR promoter. The development of transgenic rats required continuous infusion of FSH for superovulation rather than a single injection used in the development of transgenic mice,^{35,36} and a technique using miniosmotic pumps was developed for this purpose. Even using this technique, a success rate of only 7% to 14% was achieved for the production of founder animals, but without it no transgenic rats could be produced. This low rate may be because rat embryos are far less resilient than those of the mouse; they are less elastic and spongier, making it difficult to inject without damaging them, and in our experience oviduct transfers are less effective in the rat with fewer resulting pregnancies and generally smaller litter sizes. Some of the founder transgenic rats were mosaic, indicating that the transgene did not integrate into the host genome until the embryo had undergone at least one cell division, although inheritance of the transgenes in the F2 and subsequent offspring was Mendelian. The expression of the transgenes was as anticipated using the MMTV-LTR promoter, being restricted largely to the mammary glands in pregnancy,^{27,31,45} although they were also expressed at lower levels in other exocrine glands examined at the same time, notably the sebaceous and submaxillary glands, in agreement with their reported expression in certain strains of transgenic mice.⁴⁶

To our knowledge, this is the first reported transgenic model of breast cancer in which overexpression of the non-mutated human *c-erbB-2* gene induces the stochastic development of mammary carcinomas. Expression of the non-mutated rat *c-erbB-2* gene in transgenic mice also resulted in the stochastic development of mammary carcinomas, although the tumor incidence was higher (70% after 1 year) and the development of metastases

was reported in this mouse model.³² We did not find any evidence of metastases in our rat model, and we also detected a large number of benign lesions such as fibroadenomas, which were not reported in the mouse model. It is not clear at present whether our model closely mimics the human disease, in which overexpression of non-mutated *c-erbB-2* is frequently seen.^{20–22} In the wild-type rat *c-erbB-2*-expressing transgenic mouse model, the stochastic development of mammary tumors is associated with somatic mutations within the transgene itself and activation of the receptors' intrinsic tyrosine kinase activity.³³ At present, it is not known whether similar somatic mutations are responsible for the development of tumors in our rat model.

The phenotypes of hyperplasia and development of carcinomas at low frequency observed in our MMTV-TGF α transgenic rats are consistent with the transgenic mouse models in which TGF α was overexpressed in the mammary gland.^{45–48} In one of these models, the phenotype of hair loss and sebaceous gland hyperplasia was also observed.⁴⁶ Severe, pregnancy-dependent hyperplasias developed only in the MMTV-TGF α transgenic rats; MMTV-*c-erbB-2* rats did not express this phenotype. An equivalent phenotype has not been reported to develop in humans. Although a certain degree of mammary hyperplasia is always present in MMTV-TGF α transgenic rats, even during the first pregnancy, the severe, macroscopically identifiable hyperplasias that resemble lactating adenomas appeared only after five or more pregnancies and then only in approximately one-half of the animals. This suggests that either a critical level of transgene expression is needed for the severe hyperplasias or that a second, cooperating genetic event needs to take place for these hyperplasias to develop. Pregnancy-associated hyperplasias have also been described in TGF α -expressing transgenic mice, notably WAP-TGF α mice.^{48,49} Pregnancy-dependent lesions similar to those seen in our transgenic rats have also been reported in MMTV-*int-2*-expressing transgenic mice,^{50,51} in MMTV-FGF-7 (KGF)-expressing transgenic mice,⁵² and in the BR6 mouse strain.⁵³ The association with pregnancy suggests that these lesions are hormone dependent, and may possibly reflect activation of the MMTV promoter

during pregnancy. It is unclear whether these severe hyperplastic lesions can progress to hormone-independent growth, because their severity has necessitated early culling of the animals. However, as progressively growing lactating adenomas of similar histological appearance and with a similar level of TGF α expression have been observed in two of the MMTV-TGF α transgenics, it is highly plausible.

Benign fibroadenomas and other benign lesions, such as cystic expansions, sclerosing adenosis, and ductal hyperplasia, were common in the MMTV-*c-erbB-2* transgenic rats. These lesions are likely to be due to transgene expression and not spontaneous for three reasons. First, they were multifocal. Second, with the exception of one area of fibroadenoma, they were not detected in control, nontransgenic littermates of similar ages and reproductive histories. Third, the various lesions stained with antiserum to *c-erbB-2* and produced abundant quantities of transgene-specific mRNA. The frequent development of macroscopic fibroadenomas and microscopic fibroadenomatous changes appeared to be a particular property of the *c-erbB-2*-expressing transgenics; only mild fibroadenomatous changes were observed in two of the MMTV-TGF α transgenics. Moreover, whereas the fibroadenomas that developed in the MMTV-*c-erbB-2* transgenic rats stained consistently with antiserum to *c-erbB-2*, the fibroadenomatous changes observed in the MMTV-TGF α failed to stain with TGF α antiserum. This suggests that although the rat mammary gland is susceptible to the spontaneous development of microscopic fibroadenomatous lesions, expression of *c-erbB-2* greatly increases both the frequency and the size of such lesions. Fibroadenomas are also relatively common in human breasts but rarely develop in mice. However, although *c-erbB-2* is expressed in up to 30% of human breast carcinomas, it is infrequently expressed in human benign breast diseases. Therefore, it is surprising that benign lesions such as fibroadenomas develop more frequently than DCIS and invasive carcinoma in our MMTV-*c-erbB-2* transgenic rats. The reason for this is unclear. It may simply reflect a difference in the pathological consequences of *c-erbB-2* expression in the two species. However, it is possible that the temporal expression of the transgene from the MMTV-LTR promoter may be at least partially responsible, as the activity of the MMTV-LTR promoter in the virgin mammary glands of our transgenic rats is very low but is activated by hormones of pregnancy and lactation.⁵⁴ The lesions that we have observed in our transgenic rats all develop after multiple rounds of pregnancy and lactation, whereas carcinomas develop more frequently in nulliparous carcinogen-treated rats and nulliparous women. Indeed, when carcinogens are administered to parous rats where the terminal end buds of the mammary gland have already differentiated into alveolar buds they either remain unmodified, undergo dilation giving rise to hyperplastic lobules or cystic dilations, or exhibit epithelial proliferation to form benign adenomas,⁵ rather than develop carcinomas. Experiments in which the *c-erbB-2* oncogene was expressed in the reconstituted mouse mammary gland⁵⁵ further support this explanation. In that system, DCIS

developed in unmated mice but not in mated mice. In contrast, many of the mated mice developed a variety of benign lesions, such as gross hyperplasia, adenosis, and sclerosing adenosis, lesions we have commonly observed in our MMTV-*c-erbB-2* transgenic rats. The limitations on malignant development that are a probable consequence of using the MMTV-LTR promoter to drive oncogene expression suggest that to develop improved transgenic rat models for breast cancer it would be desirable to obtain a promoter active earlier in development.

The phenotypes that are common to the MMTV-TGF α and MMTV-*c-erbB-2* transgenic rats include ductal hyperplasia, failure of certain lobules to involute fully after completion of lactation, and the stochastic appearance of papillary adenomas, DCIS, and carcinomas at low frequency. The fact that benign papillary adenomatous lesions grow out of hyperplastic glands and that, where present, DCIS is invariably associated and continuous with this lesion in both *c-erbB-2*- and TGF α -expressing transgenics suggests that a progression occurs from hyperplasia to papillary adenomatous lesions and then to DCIS, which in these systems would appear to be the ultimate precursor of invasive carcinoma. Similar gradations of phenotype have been observed in human breast cancer development; for example, multiple intraductal papillomas are not infrequently associated with DCIS.⁴² Expression of either oncogene can induce a mitogenic response in rat mammary epithelium, and persistence of expression can delay or inhibit the process of involution of the gland, perhaps by preventing or slowing the process of apoptosis. However, although expression of these oncogenes predisposes the rat mammary gland to tumor development, neither oncogene alone is sufficient to induce invasive carcinomas or, for that matter, benign breast tumors. Thus, although hyperplasia or prevention of involution of the gland may be a direct result of a certain level of expression of either gene, additional genetic changes appear to be necessary for the development of tumors.

Despite the limitations discussed, the present transgenic rats provide evidence that the TGF α and *c-erbB-2* oncogenes can induce hyperplastic responses in the rat mammary gland and increase its susceptibility to tumor development. Moreover, the range of lesions that we have observed closely resembles those seen in human breasts. These transgenic rats provide a suitable baseline for studying the multistep process of mammary carcinoma development in the rat and its potential relevance to the human disease.

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